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Differential Expression of Autophagy-Related Marker (Beclin-1) in Relation to Clinicopathological Parameters in Different Breast Cancer Molecular Subtypes

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Abstract

Background: Beclin-1 plays a vital role in the vesicle nucleation in the process of autophagy and has an important role in development, tumorigenesis, and neurodegeneration.

Aim of Study: Study the role of beclin-1 and correlate the degree of its expression with available clinic-pathological data in different molecular types of breast cancer.

Material and Methods: Immunohistochemistry was performed to examine the expression of Beclin-1 different molecular subtypes of invasive ductal carcinoma and correlating it to clinicopathological parameter.

Results: The expression of Beclin-1 was decreased in breast cancer in relation to normal tissue. High expression was significantly correlated with tumor grade, distant metastasis and stage. All the cases with high Beclin-1 expression were triple negative breast cancer cases.

Conclusions: Triple negative breast cancer cases showed the highest tumor stage, grade, distant metastasis and beclin-1 expression suggesting that the overexpression of Beclin 1 may by itself confer an aggressive biological course. It could be a therapeutic target.

Key Words: *Breast carcinoma – Molecular subtypes – Autophagy, Beclin-1.*

Introduction

BREAST cancer is considered the most frequently diagnosed cancer and the leading cause of cancer mortality among women in both developed and developing countries **[1]**. In Egypt, the condition is even worse; the incidence exceeded the world records, where breast cancer accounts for about 20% of all cancers and 32.04% of female cancers diagnosed between the years 2008-2011 **[2]**. Despite recent advances in treatment protocols, it still has unfavorable prognosis with 29% mortality and 3.7: 1 incidence to mortality ratio **[3]**. This is probably due to that 12% of patients present with distant metastasis at time of diagnosis **[4,5]**.

Breast cancer is classified according to hormone receptor status (estrogen receptor (ER) status, progesterone receptor (PR) status and Her-2 neu receptor status) into four major subtypes: Luminal A, Luminal B triple positive, triple negative/basal-like and HER2 enriched type **[6,7]**.

Autophagy is a cellular component of lysosomal degradation that is subclassified as micro-autophagy, chaperone-mediated autophagy, and macro-autophagy, with macro-autophagy being the most common. This system removes and recycles the dysfunctional or damaged cellular components. This is essential for cellular homeostasis **[8]**. The process of mammalian autophagy is divided into six principal steps: Initiation, nucleation, elongation, closure, maturation, and degradation or extrusion **[8,9]**.

There are several known markers of autophagy; Among these, beclin-1 which is involved in nucleation **[10]**, Light chain (LC) 3 which participates in autophagosome formation and elongation **[11,12]** and p62, a scaffold protein that delivers ubiquitinated proteins to the autophagosome **[13,14]**.

Beclin- 1 gene maps to a tumor susceptibility locus on human chromosome 17q21 that is mono-allelically deleted in up to 40-75% of cases of sporadic ovarian and breast carcinomas **[15]**. Beclin-1 is a mammalian autophagy gene that negatively regulates tumorigenesis **[10]**.

Previous studies showed that the expression of Beclin-1 is decreased in various human cancer types **[16]**. Such as cervical **[17]**, esophageal **[17]**,

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lung [18,19], hepatocellular carcinoma [20], as well as cutaneous melanoma [20]. However, Beclin-1 expression was reported to be increased in contrast to their normal counterparts in human colon [21], gastric [21], and pancreatic cancers [22]. The difference in beclin-1 between different tissues may be explained by its dual function in both tumor inhibition and tumor progression. Some studies suggested that it is possible that different Beclin-1 complexes exist, and they can function at various cellular locations and under different stimuli [23]. This made us interested in conducting this study to examine the immunohistochemical expression of Beclin-1 in different molecular subtypes of breast cancer.

Patients and Methods

This is a retrospective study carried upon 50 specimens of Formalin-fixed, paraffin-embedded 50 patients diagnosed with invasive breast ductal carcinoma, processed between 2013 & 2015. Ten Normal control cases were taken from normal breast from patient underwent reduction mammoplasty. The Paraffin blocks were collected from the Pathology Department, faculty of Medicine and the Early Cancer Detection Unit, Benha University, Egypt. The clinicopathological data were collected from patient's records.

In each case, clinicopathologic data including the patient's age, tumor size, grade, lymph node status, distant metastasis, stage, hormonal status (ER, PR and Her-2 neu) status and ki 67 index, were obtained. The studied cases according to age, were classified into two groups <50 years and ≥50 years old groups. According to the tumor size, cases were classified into 3 groups (Up to 2cm), (>2-5cm) and (>5cm).

Histopathological study: From each selected formalin-fixed, paraffin-embedded block three sections, five microns thick were prepared. One section was stained by conventional Hematoxylin and Eosin (H&E) stain. Reviewing of histopathological type according to WHO classification (2012) and exact grading according to Elston/Nottingham modification of Bloom-Richardson system was done. This grading system is based on 3 different features (Tubule formation, nuclear pleomorphism and mitotic count) of the cells in the tumor. Each of these features is given a score of 1 to 3.

Molecular classification: Scoring of ER and PR: They were considered positive if 1% or more of tumor cells have nuclear staining of any intensity [24] while HER2 be defined as positive if 10% or more of tumor cells exhibit strong uniform membrane staining [25]. Accordingly, cases where classified into: Luminal A (ER-positive and/or PR-positive, HER2-negative), Luminal B (ER-positive and/or PR-positive, highly positive for Ki67 and/or HER2-positive), Triple negative/basal-like (ER-negative, PR negative & HER2-negative), and HER2 type (ER-negative, PR-negative & HER2-positive).

Immunohistochemical study: For immunohistochemical (IHC) staining, 10% formalin-fixed, paraffin-embedded, 4-micron tissue sections were prepared. They were immunostained for Beclin1 Rabbit polyclonal antibody at a dilution of 1: 200. DAP was utilized as a chromogen. IHC staining was performed, using detection kit according to the manufacturer data (GeneTex International Corporation). Positive control: Internal positive control was evaluated as the presence of Beclin-1 staining was detected in epithelial cells of normal ducts of the breast.

Immunohistochemical assessment:

Expression of Beclin-1 was either cytoplasmic or nucleo-cytoplasmic. Immunohistochemical cytoplasmic staining was evaluated based on both the percentage of stained cells and the immunostaining intensity. The percentage of stained cells was graded as 0 (negative), 1 (<30% positive), or 2 (>30% positive), and immunostaining intensity was graded as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong). The scores for the proportion of stained cells and the staining intensity were multiplied to provide a total score: Negative (0-1), low positive (2-4), or high positive (5-6) following the study of Won KY et al., 2010 [26].

Statistical analyses:

The collected data were summarized according to the mean ± Standard Deviation (SD), the range for quantitative data frequency, and percentage of qualitative data. Comparisons between the different study groups were carried out using the Chi-square test (χ^2) and Fisher Exact test (FET) to compare proportions as appropriate. One-way Analysis of Variance (ANOVA; F) was used to compare differences between more than two groups regarding parametric data. Statistical significance was accepted at p-value ≤0.05. The statistical analysis was conducted using SPSS (version 16).

Results

Patient characteristics:

The current retrospective study was carried on 50 cases of breast ductal carcinoma while 10 benign breast specimens were included as control cases.

According to the molecular classification; 24% of the cases were luminal A subtype, 26% of the cases were luminal B subtype, 28% of the cases were Her 2 enriched subtype and 22% of the cases were triple negative breast cancer. The relationship between the breast cancer molecular subtypes and the different clinicopathological data are shown in (Table 1). Patients with TNBC had the lowest mean age at the time of diagnosis (p=0.02) (Table 1).

Our results showed that there was a statistically significant correlation between the various molecular subtypes and tumor grade, lymph node metastasis, distant metastasis and tumor stage. Triple negative breast cancer showed higher tumor grade (p=0.03), more lymph node metastasis (p=0.03), more distant metastasis and (p=0.001) higher tumor stage (p=0.04) followed by Her-2 enriched type and the least was luminal tumors.

Table (1): Clinicopathological data among the different molecular subtypes.

Clinicopathological data	Luminal A (no.=12)	Luminal B (no.=13)	Her 2 enriched (no.=14)	Triple negative (no.=11)	Test	p-value
	No. (%)	No. (%)	No. (%)	No. (%)		
Age (years):						
Mean ±SD	51.58±10.17	58.77±7.47	52.07±9.58	45.64±11.62**	F=3.66	0.02*
Range	(34-73)	(48-70)	(37-72)	(30-65)		
Age groups:						
<50	7 (58.33)	3 (23.08)	7 (50)	8 (72.73)	X^2=6.37	0.09
>50	5 (41.67)	10 (76.92)	7 (50)	3 (27.27)		
Tumor size (cm):						
Mean ±SD	4.06±3.09	3.54±2.15	4.15±2.15	3.12±1.15	F=0.55	0.65
Range	1.5-10	1.5-10	1.5-10	1.5-5		
<2.5	8 (66.67)	4 (30.77)	3 (21.43)	3 (27.27)		
2-5	1 (8.33)	8 (61.54)	9 (64.29)	8 (72.73)	FET	0.22
>5	3 (25)	1 (7.69)	2 (14.29)	0		
Tumor grade:						
1	5 (41.67)	1 (7.69)	1 (7.14)	0	FET	0.03*
2	4 (33.33)	7 (53.85)	7 (50)	2 (18.18)		
3	3 (25)	5 (38.46)	6 (42.86)	9 (81.82)		
Lymph node metastases:						
Negative	5 (41.67)	4 (30.77)	4 (28.57)	0	FET	0.03*
1-3	4 (33.33)	6 (46.15)	2 (14.29)	1 (9.09)		
4-9	2 (16.67)	2 (15.38)	5 (35.71)	3 (27.27)		
>10	1 (8.33)	1 (7.69)	3 (21.43)	7 (63.64)		
Stage:						
I	4 (33.33)	2 (15.38)	2 (14.29)	0	FET	0.04*
II	5 (41.67)	5 (38.46)	1 (7.14)	2 (18.18)		
III	3 (25.0)	3 (23.08)	7 (50)	3 (27.27)		
IV	0	3 (23.08)	4 (28.57)	6 (54.55)		
Distant metastases:						
Absent	12 (100)	12 (84.62)	10 (71.43)	3 (27.27)	FET	0.001 **
Present	0	1 (15.38)	4 (28.57)	8 (72.73)		

* Means statistically significant.
** Means statistically highly significant.

Correlation between the expression of Beclin-1 and the clinicopathological parameters (Table 2):

Expression of (Beclin-1) was correlated with different clinicopathological findings of the studied cases. All 10 benign breast lesions specimens taken as control cases showed high positive expression for beclin-1. The current study revealed that 66% of cases were negative for Beclin1, 28% cases were low positive, and 6% percent of cases showed high positive cytoplasmic staining. We found that there was a decrease in cytoplasmic Beclin-1 expression in tumor tissue in relation to the adjacent normal tissue except for in approximately 34% of cases. Out of the positive cases; Most of the positive cases (90%) showed cytoplasmic staining while only 10% showed both nuclear and cytoplasmic staining.

There was a statistically significant positive correlation between Beclin-1 and grade (p-value =0.02), distant metastasis (p-value=0.02), stage (p-value=0.03) and molecular subtypes of IDC (p-value <0.001). The present study showed no statistically significant correlation between Beclin-1 and age of patient (p- value=0.29), tumor size (p-value=0.90) and lymph node metastasis (p-value =0.06).

Expression of Beclin-1 according to molecular subtypes:

The expression of autophagy related protein Beclin-1 in relation to different molecular subtypes is shown in Table (2). The TNBC cases had the highest positive expression, where all the cases that showed strong cytoplasmic positivity were TNBC (p=0.03). The least positivity was detected in Luminal Type A.

Table (2): Relation between Beclin-1 expression and clinicopathological parameters.

Variable	Beclin-1 expression			Test	p-value
	Negative (no.=33)	Low positive (no.=14)	High positive (no.=3)		
	No. (%)	No. (%)	No. (%)		
Age:					
Mean±SD	53.97±9.65	49.07±11.33	48.67±15.27	F=1.28	0.29
Range	(36-73)	(30-67)	(32-62)		
<50y	15 (45.45)	9 (64.29)	1 (33.33)	FET	0.50
>50y	18 (54.55)	5 (35.71)	2 (66.67)		
Tumor size (cm):					
Mean±SD	3.8±2.32	3.82±2.26	2.7±0.52	F=0.34	0.71
Range	(1.5-10)	(1.5-10)	(2.1-3)		
<2.5	11 (33.33)	6 (42.86)	1 (33.33)		
2-5	18 (54.55)	6 (42.86)	2 (66.67)	FET	0.90
>5	4 (12.12)	2 (14.29)	0		
Tumor grade:					
1	7 (21.21)	0	0	FET	0.02*
2	16 (48.48)	4 (28.57)	0		
3	10 (30.3)	10 (71.43)	3 (100)		
Lymph node metastases:					
Negative	10 (30.3)	3 (21.43)	0	FET	0.06
1-3	11 (33.33)	2 (14.29)	0		
4-9	8 (24.24)	4 (28.57)	0		
>10	4 (12.12)	5 (35.71)	3 (100)		
Stage:					
I	8 (24.24)	0	0	FET	0.03*
II	7 (21.21)	6 (42.86)	0		
III	10 (30.3)	6 (42.86)	0		
IV	8 (4.24)	2 (14.29)	3 (100)		
Distant metastases:					
Absent	24 (72.73)	12 (85.71)	1 (33.33)	FET	0.02*
Present	9 (27.27)	2 (14.29)	2 (66.67)		
Molecular subtypes:					
Luminal A	11 (33.33)	1 (7.14)	0	FET	0.03*
Luminal B	11 (33.33)	2 (14.28)	0		
Her-2 enriched	11 (30.3)	3 (21.43)	0		
TNBC	0	8 (57.14)	3 (100)		

*: Statistically significant

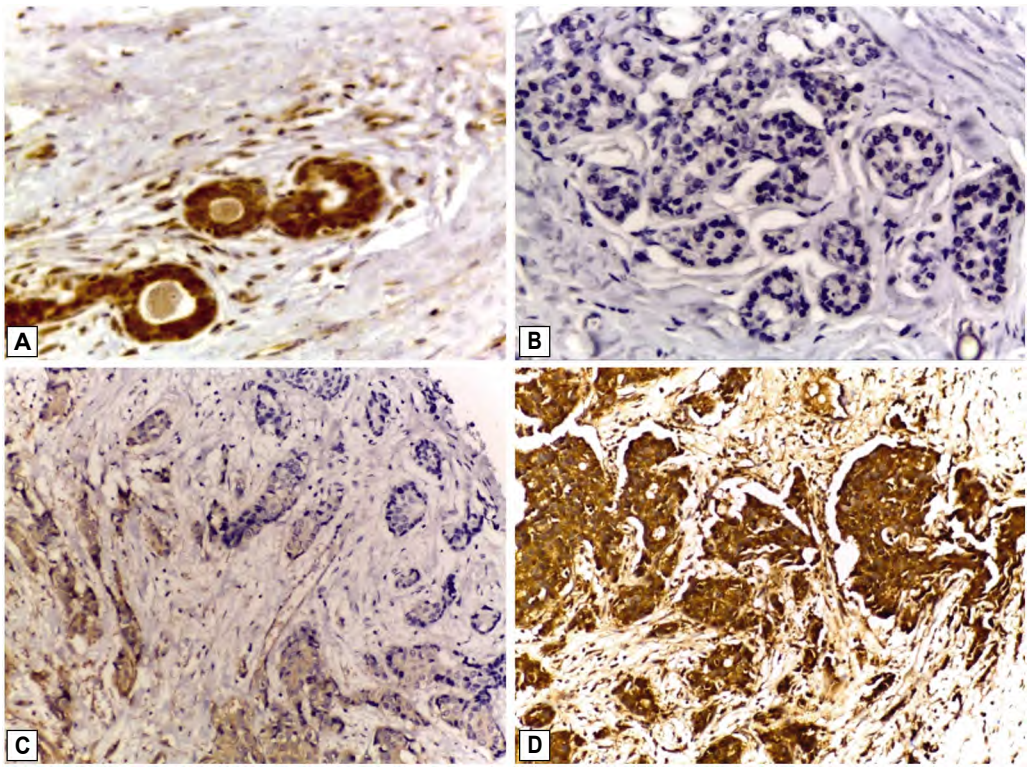

Fig. (1): Benign breast lesion showing high positive expression of Beclin-1 (A): negative expression of beclin-1 in luminal type A cancer (B): Low expression of Beclin-1 in Luminal type B (C): Strong expression of Beclin-1 in TNBC (D).

Discussion

In this study we investigated the immunohistochemical expression of autophagy related marker, Beclin-1 in different molecular subtypes of breast cancer and its correlation with the clinicopathological parameters. There are limited study relating the differential expression of autophagy related markers to clinicopathological and molecular subtypes of breast cancer. Invasive breast cancers are heterogeneous, showing distinct molecular, pathologic features and biologic behavior. Currently, the morphologic classification, histologic grade, status of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER2), along with tumor stage, are used to guide clinical management **[6]**.

Our study showed that the TNBC cases showed higher tumor grade, stage, Lymph node metastasis and distant metastasis followed by Her-2 enriched type and the least was luminal tumors as shown in (Table 1). TNBC cases showed high nuclear pleomorphism and a high mitotic index. These results were consistent with the results provided by Bauer KR et al., **[27]** and Sotiriou C et al., **[28]** who concluded that even small-sized triple negative breast tumors present a high incidence of lymph node involvement **[28]**. Also, matching the results provided by Rakha and colleagues **[29]** who proved that triple-negative phenotype was associated with distant metastasis and showed a specific pattern with high frequency of spinal cord, meninges, brain, liver and lung metastases. Visceral metastases are more common in Her-2 enriched type **[30]**, while bone metastases are a common pattern in luminal tumors **[31]**. Based on previous studies that most of triple negative cancers (83%) contain p53 and BRCA1 gene mutations while Luminal A subtype contained only 13% mutated tumors **[32,33]**. Previous studies had shown that mutations in the TP53 gene predict poor prognosis and are associated with poor response to systemic therapy **[34, 35]** this might explain the higher stage associated with TNBC in our study.

One of the mechanisms through which cancer development could be controlled is autophagy which is an important conserved catabolic process used by human cells for clearance of cytoplasmic materials, damaged organelles and aggregate-prone proteins in lysosomes. The recycling of these intracellular constituents also serves as an alternative energy source during periods of metabolic stress to maintain homeostasis and viability **[36]**. Recent evidence shows that autophagy provides a protective function for the tumor cells to limit

tumor necrosis and inflammation, and to mitigate genome damage in tumor cells in response to metabolic stress [37]. Autophagy is controlled by the combination of more than 30 autophagy related genes (Atg) [38,39]. Beclin-1 plays a crucial role in the vesicle nucleation, the formation of the double membrane of autophagosome [40] and regulation of autophagy. It has an important role in development process, aging, diabetes, and fatty liver, and tumorgenesis, so it is regarded as a potential therapeutic target [41,42]. Beclin-1 gene maps to a tumor susceptibility locus on human chromosome 17q21. It is mono-allelically deleted in most cases of sporadic human cancers, as in 75% of ovarian and 40% of prostate cancers [43,44].

Some data favor the idea that it suppresses tumor development. This was based on the studies that concluded that heterozygous disruption of Beclin-1 gene in mice displays increased proliferation and increased frequency of spontaneous malignancy and mammary neoplasia [45,46]. Whereas other data suggest that Beclin- 1 had a tumor supporter function that enhances tumor development and protects tumor cells from cell death stimuli [47]. So, Beclin-1 had a promising prognostic indicator for disease monitoring.

The current work demonstrated that all 10 benign breast specimens showed high cytoplasmic positive expression for Beclin-1. While of cancer cases; 66% were negative and 28% were weak positive while only 6% showed strong positivity. All the strong positive cases (6%) were TNBC. A decrease in cytoplasmic Beclin-1 expression in tumor tissue in relation to the adjacent normal tissue was observed. This supports the hypothesis that it suppresses tumor development. This was also reported in different tissues. The expression of Beclin-1 was also observed to be decreased in various human cancer types, such as hepatocellular carcinoma [20], esophageal carcinomas [48], cutaneous melanoma [49], cervical carcinoma [17] and lung cancers [19]. Also, a study held on canine mammary tumors proved that the cytoplasmic expression of Beclin-1 in cancer cells was lower than that of normal mammary glands [50]. Cell culture technique proved that Beclin 1 depletion in monolayer cultures was found to increase tumor genesis and progression [51]. However, Beclin-1 expression was reported to be increased in human colon, gastric [21], and pancreatic cancers [22] in contrast to their normal counterparts. This supports that Beclin-1 acts as a tumor suppressor gene involved in the tumor initiation step [52].

The cytoplasmic location of Beclin-1 (Fig. 1) is explained by its importance in formation of the double membrane of autophagosome in the process of autophagy and serves as a scaffold by binding to other proteins. This takes place in the cytoplasm. Beclin-1 is reported to reside in the trans Golgi network, endoplasmic reticulum and the mitochondria [53,47]. However, nuclear beclin- 1 expression is due to presence of its regulatory gene (BECN-1) [54]. The shift in expression might be related to the loss of Beclin-1 gene suppressor function [55, 65]. This agrees with the results provided by Won et al., and Qu X [26,43] who stated that the Beclin-1 gene is mono-allelically deleted in majority of cases of sporadic ovarian and breast carcinomas that can lead to defects in autophagy and apoptosis, contributing to tumorigenesis. Morikawa et al., [43] also reported that many breast cancer cells lack beclin- 1 expression due to a combination of monoallelic deletion and epigenetic silencing of the Beclin-1 gene.

The current study showed a statistically significant correlation between Beclin- 1 and grade of the tumor (p-value=0.02). High positive expression of Beclin-1 was associated with grade III breast carcinoma. This agrees with the results provided by several studies [57,58]. However, Choi and colleagues, [59] demonstrated no significant correlation between Beclin-1 and the grade of the tumor. On the other hand, other studies [26] demonstrated an inverse significant correlation between Beclin- 1 and the grade of the tumor. High tumor grade has central necrosis which stimulates autophagy and so high Beclin- 1 expression to facilitate removal of the necrotic tissue [37]. This might explain that TNBC cases show the highly positive Beclin-1 expression (Fig. 1).

Beclin- 1 is correlated with distant metastasis (p-value=0.02) and with high TNM stage of breast carcinoma (p-value=0.03). This could be explained by the loss of extracellular matrix attachment and lack of appropriate matrix contact induces autophagy to promote cell survival, either during early carcinoma formation or in the later stages of dissemination and metastasis [60]. Also, autophagy tends to facilitate metastatic process by sustaining spreading cell survival and colonization at a secondary site and by inducing these cells to enter dormancy if they fail to establish stable contact with the extracellular matrix in the new environment [61]. The high expression of Beclin-1 is associated also with high stage of endometrial carcinoma [62] and gastric carcinoma [63]. The correlation between Beclin-1 expression and the advanced grade and stage of the tumor could be explained

by the observation that Beclin-1 has a predominantly tumor suppressor function in the initial tumorgenesis phase, and a predominantly tumor supporter function thereafter **[40]**. There was no correlation between beclin-1 expression and tumor size or age of patient and this agrees with the study of Choi, et al., **[59]**.

Regarding the molecular subtypes of IDC, our study reported a high statistically significant correlation between Beclin-1 expression and molecular subtype of IDC (p-value <0.001). Cytoplasmic Beclin- 1 expression was lowest in luminal breast tumors. While cytoplasmic Beclin-1 expression was highest in TNBC tumors which have high tumor grade (Tables 1,2). This was in agreement with the results of several other studies **[59,64]**. However, it is worth noting that allelic deletion of Beclin-1 and depressed Beclin-1 expression exist in some TNBC cell lines **[65]**. Our results could be explained by the higher level of hypoxia in TNBC due to central necrosis and high tumor grade than in other breast cancer subtypes. Hypoxia is a well-known stimulus for inducing autophagy which is an adaptive metabolic response necessary to maintain homeostasis and cell survival **[66]**.

In conclusion, Triple negative breast cancer cases showed the highest tumor stage, grade, distant metastasis and beclin-1 expression suggesting that the overexpression of Beclin-1 may by itself confer an aggressive biological course. It could be a therapeutic target.

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التعبير التفاضلى للدلالة المناعية ذات الصلة بالإلتهام الذاتى (بكلين-١) ومقارنته بالعوامل الإكلينيكية الباثولوجية فى مختلف الأنواع الجزيئية لسرطان الثدى

يصنف سرطان الثدى وفقاً لحالة مستقبلات الهرمونات (مستقبلات الأستروجين والبروجستيرون والهير-٢) إلى أربعة أنواع: الطراز القنى (أ) إيجابى لهرمون الأستروجين والبروجستيرون وسلبىلهير-٢، الطراز القنى (ب) إيجابى لهرمون الأستروجين والبروجستيرون وإيجابى للهير-٢، الثلاثى السلبى (سلبى للأستروجين والبروجستيرون وسلبى هير-٢)، ولكل منها أهمية فى المظاهر السريرية، والإستجابة للعلاج.

الإلتهام الذاتى يلعب دوراً هاماً فى الخلايا السرطانية وكذلك الخلايا الطبيعية. ويعتبر الإلتهام الذاتى سلاح نو حدين. بكلين-١ هو البروتين المشفر بواسطة جين بكلين-١. وهو يشارك فى تظيم الإلتهام الذاتى وله دور مهم فى عملية تنمية ونشأة الورم. يلعب دورا حاسماً فى التنوى فى عملية الإلتهام الذاتى.

يهدف هذا البحث إلى دراسة علاقة بكلين-١ ودرجة ظهوره مع البيانات الإكلينيكية فى مختلف أنواع سرطان الثدى.

وفى هذه الدراسة وجد أن النوع الثانى السلبى لسرطان الثدى كان الأكثر عدوانية مقارنة بالأنواع الأخرى كما وجد أن الدلالة المناعية بكلين-١ تنخفض فى سرطان الثدى. كذلك أوضحت الدراسة وجود علاقة إحصائية طردية بين بكلين-١ ودرجة الورم وتكوين الثانويات ومرحلة الورم ومختلف الأنواع الجزيئية.

ونستنتج من هذه النتائج أن الإفراط فى ظهور بكلين-١ فى حد ذاته ينبئ بالطبيعة البيولوچية العدوانية للورم. لذلك يعتقد أنه يمكن إستخدام مثبطات بكلين-١ لزيادة فاعلية العلاج الكيميائى فى سرطان الثدى.